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13. ABSTRACT (Maximum 200 Words) Recently we identified two prostate-specific enhancers, AREc3 and PSME(del2), from PSA gene and PSMA gene, respectively, and generated a chemic promoter, called PSES, by combining AREc3 and PSME(del2). The goal of this research is to use AREc3/PSME(del2) or PSES to develop a prostate-restricted replicative adenovirus (PRRA) and to arm PRRA with an apoptosis inducer, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand). We constructed 3 PRRAs according to our original proposal. None of them demonstrated a good prostate specificity. We, then, constructed another PRRA, called AdE4PSESE1a, using a different strategy. AdE4PSESE1a only replicated in PSA/PSMA positive prostate cancer cells or cells expressing adenoviral E1 and E4 proteins. Then, we armed AdE4PSESE1a with TRAIL to make AD-IU-2 as our original plan. Ad-IU-2 retains prostate specificity of AdE4PSESE1a and expresses TRAIL on in PSA/PSMA positive cells. Ad-IU-2 demonstrated a better in vitro cell killing activity and tumor killing activity in vivo than AdE4PSESE1a against PSA/PSMA positive prostate cancers. We are on the process of arming AdE4PSESE1a with a soluble TRAIL and creating a hybrid tumor model containing both PSA/PSMA-positive and negative prostate cancer cells to test the therapeutic advantage of TRAIL-armed PRRA.				
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INTRODUCTION

Metastatic human prostate cancer (PC) is commonly treated by hormone, radiation, and/or chemotherapy. Inevitably, these patients will eventually relapse and develop androgen-independent disease with osseous metastasis. Since no effective therapy is presently available for the treatment of PC metastasis, we are developing a novel gene therapy modality for hormonal refractory prostate cancer based on several prostate specific enhancer cores, AREc3, PSME(del2) and PSES, generated in my laboratory. In this study, we proposed to generate an apoptosis inducer, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand), armed prostate restricted replicative adenovirus (PRRA) to treat androgen-independent prostate cancers. Specific Aim 1 will continue our examination into whether PSES chimeric enhancer retains prostate specific activity useful for driving the expression of therapeutic gene to treat AI prostate cancers. Specific Aim 2 will construct several PRRA's based on AREc3/PSMEdel2 or PSES enhancers and investigate their tissue specificity and tumor-killing activity. Specific Aim 3 will arm PRRA with apoptosis inducer, Trail, to enhance the tumor killing activity of PRRA..

BODY

Task 1. To test tissue-specificity of the PSES chimeric enhancer. (Months 1-12):

- a. Test the tissue-specificity of Ad-PSES-Luc in tissue culture cells (Months 1-8).

This is ongoing.

- b. Test the tissue-specificity of Ad-PSES-Luc in animals (Months 9-12).

Androgen-independent CWR22rv prostate tumors were established in nude mice and treated with 5.7×10^9 virus particles of Ad-CMV-luciferase (■) or Ad-PSES-luciferase (□) via tail vein injection. After 2 days, tumors and organs were harvested from sacrificed mice and homogenized. Protein extract was used for luciferase assay. Three mice were used for each group.

Ad-CMV-Luc demonstrated high activity in tumor, and low activity in liver, lung and spleen. Kidney and prostate showed the lowest activity in all organs tested. This result is consistent with the reported virus distribution study and our prior results. As expected, Ad-PSES-Luc also has high activity in tumor, and its activity in liver, lung and spleen is much lower than Ad-CMV-Luc. The activity of Ad-PSES-Luc in prostate and kidney is similar to the background signal

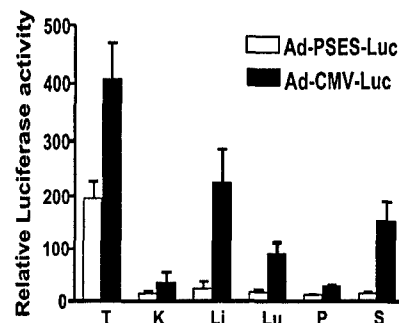


Figure 1. D. 5.7×10^9 virus particles of Ad-CMV-luciferase (■) or Ad-PSES-luciferase (□) were injected into the tail vein of mice. After 2 days, tumors and organs were harvested from sacrificed mice and homogenized. Protein extract was used for luciferase assay. Three mice were used for each group. Results are presented as mean \pm S.D. Luciferase activity was presented as per mg protein. T: tumor; K: kidney; Li: liver; Lu: lung; P: prostate; S: spleen.

Task 2. To investigate the capability of PSA and PSMA enhancer to drive adenovirus replication in a prostate cancer-specific manner (Months 1-24):

- a. Construct and amplify AREc3/PSME(del2) restricted replicative adenoviruses (Months 1-8).

We generated an AREc3/PSME(del2) restricted replicative adenoviruses, AdE4PSESE1a, by putting the PSES and E1a gene in the E4 region (see **Figure 1 in attached paper**). A CMV-EGFP expression cassette was inserted in front of the E1b gene.

- b. In vitro test tissue-specific expression of E1a and E1b by AREc3/PSME(del2) restricted replicative adenoviruses (Months 9-12).

AdE4PSESE1a derived E1a and E4 expression on in PSA/PSMA positive cells (see **Figure 4 in attached paper**).

- c. In vitro test tissue-specific replication of AREc3/PSME(del2) restricted replicative adenoviruses (Months 11-14).

We then tested the tissue specific activity of AdE4PSESE1a on several PSA/PSMA-positive and negative cells. Cells were infected with AdE4PSESE1a and monitored daily under a fluorescent microscope up to 5 days. EGFP expression represented AdE4PSESE1a infection. At 1 day after infection, EGFP-expressing cells were easily detected in all cell types tested (see **Figure 5 in attached paper**). The number and intensity of green cells increased in PSA/PSMA-positive prostate cancer cells, but not in PSA/PSMA-negative cells. At day 5, a comet-like patch of green cells had formed only in PSA/PSMA-positive prostate cancer cells. At the same time, we could detect virus plaques under a light microscope. This result and a viral replication assay (see **Table 1 in attached paper**) clearly demonstrate that AdE4PSESE1a only replicates efficiently in cells either providing endogenous E4 gene products, such as 911E4 cells, or allowing E4 expression from the Ad genome, such as C4-2 cells. This established as a proof of principle that the E4 gene can be used to control Ad replication.

- d. In vitro test the therapeutic efficacy of AREc3/PSME(del2) restricted replicative adenoviruses (Months 13-16).

To test the tissue/tumor specific killing activity of AdE4PSESE1a, serial dilutions of AdE4PSESE1a and Ad-wt were applied to C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, A549, MCF10A and HEK293 in 96-well plates. Cells were monitored under the microscope daily. AdE4PSESE1a was able to kill PSA/PSMA positive C4-2 and CWR22rv at the same doses as Ad-wt. On the

other hand, AdE4PSESE1a required 100-fold more virus than Ad-wt to kill LoVo, 500-fold more to kill DU145 and HeLa, 1,000-fold more virus to kill PC-3 and MCF10A, and 10,000-fold more viruses to kill HEK293 cells. This result indicates that the killing activity for AdE4PSESE1a was the same as Ad-wt for PSA/PSMA-positive cancer cells, and significantly attenuated for PSA/PSMA-negative prostate cancer cells and non-prostatic cancer cells. (see **Figure 6 in attached paper**).

- e. In vivo test the therapeutic efficacy of AREc3/PSME(del2) restricted replicative adenovirus (Months 17-22).

CWR22rv subcutaneous tumors were established in athymic nude mice. The mice were castrated three days after CWR22rv inoculation to test whether AdE4PSESE1a was able to eliminate AI tumors in a castrated host. Mice were randomized 3 weeks after cell inoculation (9 tumors from 6 mice in each group) and received intra-tumoral injections of 2×10^7 ifu (determined by Adeno-XTM Rapid Titer System from Invitrogen) of AdE4PSESE1a or AdCMV-EGFP (a replication-deficient adenovirus used as a negative control). In addition, 7 tumors from 6 mice were treated intravenously with 5×10^7 ifu of AdE4PSESE1a twice at a one-week interval. Tumor sizes were monitored once a week. The result demonstrates that tumor growth was significantly retarded in the AdE4PSESE1a-treated groups regardless of the route of viral injection, compared to those in the AdCMV-EGFP-treated group (see **Figure 7 in attached paper**). We frequently observed complete responses to AdE4PSESE1a therapy. For example, 2 tumors disappeared in the AdE4PSESE1a intra-tumoral injection group (see **Figure 7 in attached paper**), and 3 tumors disappeared in the tail vein injection group (see **Figure 7 in attached paper**) in this study. However, the growth inhibitory effects were temporally, and the majority of tumors eventually grew back even in animals treated via tail vein injection. (see **Figure 7, 8 and 9 in attached paper**).

Task 3. To Arm AREc3/PSME(del2) restricted replicative adenovirus with soluble Trail (Months 13-34): **On going.**

- a. Construct and amplify Trail-armed AREc3/PSME(del2) restricted replicative adenovirus (Months 13-18).
- b. In vitro test the therapeutic efficacy of Trail-armed AREc3/PSME(del2) restricted replicative adenovirus. (Months 19-22).
- c. In vivo test the therapeutic efficacy of Trail-armed AREc3/PSME(del2) restricted replicative adenovirus. (~222 animals will be used) (Months 23-28).
- d. Establish a chimeric tumor model with both PSA/PSMA positive and negative prostate cancer cells. (~100 animals will be used) (Months 25-28).
- e. In vivo test the bystander killing effect of Trail-armed AREc3/PSME(del2) restricted replicative adenovirus. (~66 animals will be used) (Months 29-34).

Task 4. To organize data for report: (Months 35-36)

f. KEY RESEARCH ACCOMPLISHMENTS

1. Successfully construct AdAREc3E1aPSME(del2)E1b, AdPSME(del2)E1aAREc3E1b and AdE1aPSESE1b. We also made another construct, AdE4PSESE1a, which is not described in the original proposal.
2. We finished the study on prostate specific activities of AdAREc3E1aPSME(del2)E1b, AdPSME(del2)E1aAREc3E1b, AdE1aPSESE1b and AdE4PSESE1a.
3. We have pulished our studies on AdE4PSESE1a.
4. We have successfully made a TRAIL-armed PRRA.

REPORTABLE OUTCOMES

Some of the results of this study have been published.

Li, X, Y Zhang, H-S Kim, K-H Bae, KM Stantz, S-J Lee, C Jung, JA Jiménez, M-H Jeng, TA Gardner, **C Kao**. Gene therapy for prostate cancer by controlling adenovirus E1A and E4 gene expression with PSES enhancer. Cancer Res. 65:1941-1951, 2005.

CONCLUSIONS

In this past year we have successfully construct a PRRA, AdE4PSESE1a, in addition to those proposed in the original proposal. AdE4PSESE1a is the only PRRA demonstrated a replication activity comparable to a wild-type adenovirus, and retained strong prostate specificity. It only replicated and killed PSA/PSMA-positive prostate cancers as expected. We further armed AdE4PSESE1a with TRAIL to make AdIU2 as originally planned. We are on the process of making AdIU2' which will be armed with a soluble TRAIL.

REFERENCES

Gene Therapy for Prostate Cancer by Controlling Adenovirus *E1a* and *E4* Gene Expression with PSES Enhancer

Xiong Li,^{1,5} Yan-Ping Zhang,^{1,5} Hong-Sup Kim,^{1,5} Kyung-Hee Bae,^{1,5} Keith M. Stantz,³ Sang-Jin Lee,^{1,5} Chaeyong Jung,^{1,5} Juan A. Jiménez,^{1,2,5} Thomas A. Gardner,^{1,2,5} Meei-Huey Jeng,^{2,4,5} and Chinghai Kao^{1,2,5}

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Abstract

PSES is a chimeric enhancer containing enhancer elements from prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) genes that are prevalently expressed in androgen-independent prostate cancers. PSES shows strong activity equivalent to cytomegalovirus (CMV) promoter, specifically in PSA/PSMA-positive prostate cancer cells, the major cell types in prostate cancer in the absence of androgen. We developed a recombinant adenovirus (Ade4PSESE1a) by placing adenoviral *E1a* and *E4* genes under the control of the bidirectional enhancer PSES and enhanced green fluorescent protein gene for the purpose of intratumoral virus tracking under the control of CMV promoter. Because of PSES being very weak in nonprostatic cells, including HEK293 and HER911 that are frequently used to produce recombinant adenovirus, Ade4PSESE1a can only be produced in the HER911E4 cell line which expresses both *E1* and *E4* genes. Ade4PSESE1a showed similar viral replication and tumor cell killing activities to wild-type adenovirus in PSA/PSMA-positive prostate cancer cells. The viral replication and tumor cell killing activities were dramatically attenuated in PSA/PSMA-negative cells. To test whether Ade4PSESE1a could be used to target prostate tumors *in vivo*, CWR22rv s.c. tumors were induced in nude mice and treated with Ade4PSESE1a via intratumoral and tail vein injection. Compared to tumors treated with control virus, the growth of CWR22rv tumors was dramatically inhibited by Ade4PSESE1a via tail vein injection or intratumoral injection. These data show that adenoviral replication can be tightly controlled in a novel fashion by controlling adenoviral *E1a* and *E4* genes simultaneously with a single enhancer. (Cancer Res 2005; 65(5): 1941-51)

Introduction

Prostate cancer is the most frequently diagnosed cancer in men in the United States, with an estimated incidence of more than 916 new cases and 115 deaths per day. Frequently, patients present with locally advanced disease and/or detectable distant bone metastases at initial diagnosis. The best available treatment for patients with advanced disease is androgen ablation therapy, based on the observations of Huggins and Hodges (1) that clinical prostate cancer is under the trophic influence of male hormones. Tumor regression and improvement of clinical symptoms are

temporary and inevitably the disease progresses to an androgen-independent state. Currently, no curative therapy is available for androgen-independent prostate cancers.

Gene therapy provides an attractive opportunity to target androgen-independent prostate cancer. Unlike traditional chemotherapy, it can be designed and customized to target cancers specifically according to our understanding of the disease at a molecular level. We have shown that prostate-specific antigen (PSA) or osteocalcin promoter and human herpes simplex thymidine kinase gene-based therapy inhibits the growth of androgen-independent PSA-producing cells (2-4); however, a number of restrictions limit the efficacy of this type of gene therapy. For example, this therapy only works in proliferating tumor cells and is ineffective for slow-growing cancers like prostate cancer. We further explored prostate-restricted replicative adenoviruses as an aggressive approach to eliminate prostate metastases (5). Compared to the replication-deficient adenovirus, this approach allows the virus to propagate and infect more cells in the tumor mass, which will improve the inadequate *in vivo* infectivity and biodistribution of adenovirus.

The most commonly used strategy to construct a tissue/tumor-restricted replicative adenovirus (TRRA) is to place the adenoviral *E1a* gene under the control of tissue/tumor-specific promoters (5-7). *E1a* protein is central to the regulation of adenoviral gene expression and viral replication by inactivating the function of tumor suppressor pRB and transactivating late gene promoters. Theoretically, without *E1a* protein the expression of late gene products would be diminished, preventing propagation of the virus in the cell. Tissue/tumor-specific replication could be achieved by placing *E1a* gene under the control of tissue/tumor-specific promoter; however, leaky replication is frequently observed with this strategy. Controlling both *E1a* and *E1b* genes with a tissue/tumor-specific promoter further improves the tissue/tumor-specific replication of TRRAs (8, 9). Recent studies showed tight regulation of adenoviral replication by placing *E1a* and *E4* genes under the control of two promoters that are either duplicates or distinct (10, 11). Because of the difficulty in finding two active and tightly regulated promoters for a tumor type, and the use of two copies of the same promoter might induce recombination, we developed a new strategy which places both *E1a* and *E4* genes under the control of one single prostate-specific enhancer, PSES.

PSES contains enhancer elements from PSA and prostate-specific membrane antigen (PSMA) genes, the two best studied antigens expressed by the majority of androgen-independent prostate cancers. Delineating the regulatory mechanism of PSA and PSMA expression in androgen-independent prostate cancers, we found that the main prostate-specific enhancer activity of the

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PSA enhancer core lies within a 189-bp region called AREc3 which is located 4.2 kb upstream of the start codon (12). The main prostate-specific enhancer activity of the PSMA enhancer core lies within a 331-bp region located in the third intron of the PSMA encoding gene, called PSME(del2) (12). PSES, a combination of AREc3 and PSME(del2), showed much stronger transcriptional activity than either AREc3 or PSME(del2) alone in the presence or absence of androgen and retained tight prostate-specific activity *in vitro* and *in vivo* with an activity comparable to cytomegalovirus (CMV) promoter in PSA/PSMA-positive cells and a basal activity in all PSA/PSMA-negative cells from a variety of organ tissues (12). We believe that PSES is a better transcriptional regulator than AREc3 and PSME(del2) for developing a prostate-specific replication-competent adenovirus for patients with androgen-independent cancers.

Materials and Methods

Cells and Cell Culture. HEK293 is a transformed human embryonic kidney cell line established by Graham (13) that expresses complementing adenoviral E1 protein supporting the replication of E1-deleted recombinant adenoviruses. HEK293 was maintained in MEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% MEM nonessential amino acids. HER911 is a human embryonic retinoblast cell immortalized with a plasmid containing adenoviral E1 gene (bp 79-5,789 of the Ad5 genome). HER911 was cultured in DMEM, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (14). HER911E4 is an HER911 derivative that expresses adenoviral E4 protein under the control of *tetR* (15). HER911E4 was maintained in the same medium as HER911 supplemented with 0.1 mg/mL hygromycin B (Calbiochem, San Diego, CA) and 2 µg/mL doxycycline (Sigma, St. Louis, MO). C4-2 is an androgen-independent human prostate cancer cell line derived from LNCaP that retains the expression of androgen receptor, PSA, and PSMA (16). CWR22rv is an androgen-independent prostate cancer cell line derived from an androgen-dependent human xenograft tumor, CWR22s (17). PC-3 is an androgen-independent, androgen receptor-, and PSA-negative human prostate cancer cell line derived from the bone marrow aspirates of a patient with confirmed metastatic disease (18). DU145 is an androgen-independent, androgen receptor-, and PSA-negative human prostate cancer cell line established by Stone et al. (19) from a patient with prostate cancer brain metastases. C4-2, CWR22rv, PC-3, and DU145 were all maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin/

streptomycin. MCF10A, a nontumorigenic human mammary epithelial cell line, was cultured in a 1:1 mixture of DMEM and F12 medium (DMEM-F12) in which 500 mL of medium were supplemented with 26.3 mL horse serum (Invitrogen), 500 units penicillin/streptomycin, 0.5 mol/L L-glutamine, 5.36 mg insulin, 10.75 µg EGF, 52.5 µg cholera toxin, and 250 µg hydrocortisone (20). HeLa, a human cervical carcinoma, was maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (21). LoVo, a colon cancer cell (22), was maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were refed two to three times per week with fresh growth medium and maintained at 37°C in a 5% CO₂ incubator.

Construction of Recombinant Adenovirus. The recombinant adenovirus construction strategy is based on a system developed by Dr. Xavier Danthine (O.D.260, Inc., Boise, ID). The system comprises the cloning vector, pAd1020SfIdA, containing the adenoviral left ITR and packaging signal (bp 1-358) and the adenoviral genome vector, pAd288, containing the right arm of the adenoviral genome from 3,504 bp to the end with the E3 region. pAd288 was modified by inserting the *E1b* gene (including E1b TATA box, bp 1,672-3,503) to make pAd288E1b. The right end of Ad5 [from bp 35,464 (*PacI*) to 35,938 (*AvrII*)] was cloned from pAd288 into pBKS II (Stratagene, Cedar Creek, TX). The E4 enhancer region [from bp 35,641 (*PacI*) to 35,822 (*AvrII*)] was replaced by a synthetic multiple cloning site made by PCR. Then, PSES-E1a [from bp 468 (E1a TATA box) to 1,644] was cloned into the multiple cloning site. The modified *PacI*/*AvrII* fragment was cloned back into pAd288E1b to make pAd288E1b-E4PSESE1a. A CMV-EGFP expression cassette was cloned into pAd1020SfIdA to make pAd1020SfIdA-CMV-EGFP, which was then digested with *SfiI* to release CMV-EGFP together with the adenoviral left ITR and packaging signal (bp 1-358) and a kanamycin resistance gene (*Kan^r*). The *Kan^r*-ITR-EGFP-CMV fragment was then cloned into *SfiI*-digested pAd288E1b-E4PSESE1a. The ligated DNA was transformed into *E. coli* cells, which were then plated onto an agar plate containing both ampicillin and kanamycin. Cosmid DNA was purified, digested with *PacI* to release adenoviral genome, and transfected into HER911E4 (~80% confluency) using a Lipofectamine 2000 transfection reagent (Invitrogen) to generate the recombinant adenovirus, AdE4PSESE1a. The total length of the recombinant viral genome is 37,821 bp. Its structure is illustrated in Fig. 1. The CMV-EGFP expression cassette in the viral genome allows us to monitor the viral propagation *in vitro* and distribution of the virus *in vivo* because enhanced green fluorescent protein (EGFP), as a marker of viral infection, can infer viral replication indirectly. The plate was incubated at 37°C under 5% CO₂ for 7 to 10 days after transfection to allow for sufficient cytopathic effect. Then AdE4PSESE1a was amplified in HER911E4 from one P60 dish to one T75 flask, to triple-flasks, and finally to cell factories. The adenovirus was purified by CsCl

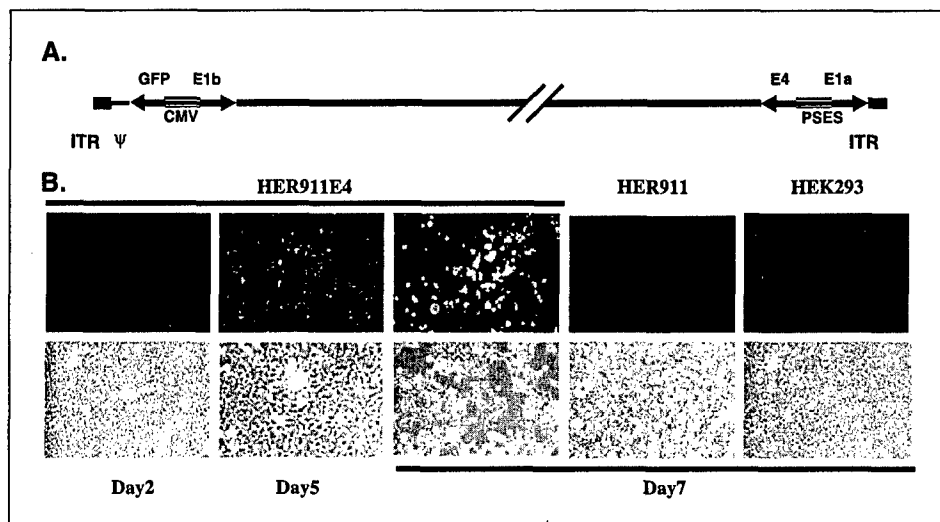


Figure 1. A, structure of AdE4PSESE1a. Adenoviral *E1a* gene was placed at the right end of the adenoviral genome to avoid potential interference from the adenoviral packaging signal. PSES is employed to direct the expression of *E1a* and *E4* genes. A CMV promoter-controlled EGFP expression cassette is cloned into the left end of the adenoviral genome to allow the tracking of intratumoral adenovirus. B, generation of AdE4PSESE1a. The viral genome was released from the cloning vector by *PacI* and transfected into helper cells, HEK293, HER911, and HER911E4, using Lipofectamine 2000 transfection reagent. Green fluorescent cells were observed daily until the formation of plaques seen by fluorescence microscopy. The cells were observed until cytopathic effect formation under light microscopy.

gradient centrifugation. All gradient-purified viral stocks were then dialyzed against dialysis buffer (1,000 mL dialysis buffer contains 789 mL double-distilled water, 1 mL 1 mol/L $MgCl_2$, 10 mL 1 mol/L Tris-HCl (pH 7.5), and 200 mL 50% glycerol) for 24 hours at 4°C, with three buffer changes. Aliquots of purified and dialyzed viruses were stored at -70°C for future use. The viral titer was determined by Adeno-XTM Rapid Titer System (Invitrogen) according to the instructions of the manufacturer. This kit detects the adenovirus capsid hexon protein by immunohistochemistry. Final titer was expressed as infection forming units (IFU). The viral DNA was purified by phenol/chloroform extraction and was used as a template for PCR. Four PCR primers were chosen within the E4-PSES-E1a gene cassette including E4 reverse (primer 1: ACCACTCGAGCCTAGGCAAAA-TAGCACCTT), PSES reverse (primer 2: AGTACTCCGATGACGTAATAAGT-CATAT), PSES forward (primer 3: GGAGGAACATATTGTTATTCGA), and E1a reverse (primer 4: CGGGAAAAATCTGCGAAACC). PCR was done with an initial denaturation step of 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute, with extension in the last cycle lasting for 10 minutes. The PCR products were analyzed on a 1% agarose gel by electrophoresis.

Adenoviral Infectivity Assay. C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, MCF10A, and HEK293 cells were seeded (2.5×10^5 cells per well) in a 12-well plate 1 day before viral infection. Cells were infected with serial doses of AdCMV-Luc (an E1/E3-deleted recombinant adenovirus carrying the luciferase reporter gene controlled by CMV promoter) from 0.05 to 37.25 multiplicity of infection (1 multiplicity of infection = 1 IFU per cell). The media were changed 24 hours after addition of the virus. The cells were harvested 48 hours after infection for luciferase assay (Promega, Madison, WI). The luciferase activities obtained were used as a reference to adjust the virus titer for each cell line to obtain a similar infectivity for the experiments. The accuracy of the above titer assay was checked by reinfecting cells with the amount of virus derived from the assay.

Western Blot Analysis. C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, and MCF10A cells were seeded in six-well plates and infected with AdE4PSESE1a and a wild-type adenovirus (Ad-wt) 1 day after cell seeding. Each cell line was infected with different amounts of virus according to the luciferase activity obtained above to achieve similar infectivities. The cells were washed with cold PBS and lysed with radioimmunoprecipitation assay buffer [1 mL modified radioimmunoprecipitation buffer supplemented with 2.5 μ L proteinase inhibitors (Sigma) and 20 μ L 57 mmol/L phenylmethylsulfonyl fluoride] 24 hours after infection. Cells were collected into Eppendorf tubes and incubated on ice for 1 hour. Cell debris was spun down and the supernatant was kept at -70°C. Protein concentration was evaluated by the Bradford protein assay. The same amount of protein (5 μ g) collected above was subjected to SDS-PAGE separation and electroblotted to a nitrocellular membrane using a NOVEX gel system (Invitrogen). SeeBlue marker (Invitrogen) was used as a molecular weight indicator. The membrane was probed with an anti-adenovirus-5 E1a antibody (BD Biosciences PharMingen, San Diego, CA), followed by a horseradish peroxidase-conjugated anti-mouse immunoglobulin G secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used to detect the signal.

Reverse Transcription-PCR for E4 mRNA. CWR22rv, C4-2, PC-3, DU145, HeLa, LoVo, and MCF10A cells were seeded in P100 dishes (5×10^6 cells per dish) and infected with AdE4PSESE1a and Ad-wt 1 day after cell seeding (~80% cell confluency). Each cell line was infected with different doses of virus to achieve comparable infectivity as described above. Cells were washed once with cold PBS and lysed for RNA isolation using TRIzol reagent (Invitrogen). Cell lysates were scraped, collected in a 1.5 mL Eppendorf tube, pipetted up-down several times, and extracted with 200 mL chloroform. The aqueous phase was removed to a new Eppendorf tube and the mRNA was precipitated with isopropanol. Final total mRNA was dissolved in 30 μ L diethyl pyrocarbonate-treated H_2O and its concentration was measured at A_{260} . Potential contamination of genomic DNA was checked by PCR using no reverse transcriptase as control. The RNeasy Mini kit (Qiagen, Valencia, CA) was used to purify mRNA if DNA contamination

was identified. Reverse transcription-PCR was done using a kit from Invitrogen. The PCR products were analyzed on a 1% agarose gel by electrophoresis. Actin expression by reverse transcription-PCR was used as an internal standard of RNA loading in each sample.

Viral Replication Assay. C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, MCF10A, and HEK293 cells were seeded in six-well plates (1×10^6 cells per well) 1 day before viral infection and subsequently infected with AdE4PSESE1a or Ad-wt. Each cell line was infected with standardized doses of virus as described above. The media were changed 24 hours later, and the viral supernatants were harvested 3 days after the infection. The cells were examined under the microscope daily for up to 5 days. Then, the titers of the harvested virus soups were checked by titer assay. HER911E4 cells were seeded in 96-well plates (5×10^3 cells per well) 1 day before viral infection. The cells were infected with serial volume dilutions of the harvested supernatants, ranging from 1 to 10^{-11} μ L per well. A row of eight wells was used for each dose. The media were changed on day 4, and the cells were examined under the microscope on day 7. The doses of the produced viruses were shown as an LD₅₀ value (the dilution factor that causes a cytopathic effect in at least four wells of cells in a row on a 96-well plate on day 7). A tissue-specificity index was obtained by dividing the LD₅₀ value of therapeutic viruses to that of wild-type.

Cell Killing Assay. C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, MCF10A, and HEK293 cells were seeded in 96-well plates (5×10^3 cells per well) 1 day before infection. The cells were infected with serial doses, ranging from 50 to 5×10^{-9} multiplicity of infection of AdE4PSESE1a and Ad-wt. A row of eight wells was used for each dose. The media were changed on day 4, and the cells were examined under a light microscope on day 7. The viral killing activity was represented as an LD₅₀ value. A killing activity index was obtained by dividing the LD₅₀ value of therapeutic viruses to that of wild-type. The value is expressed as a log₁₀ scale, such that a value of 0 indicates the therapeutic virus has the same killing activity as wild-type virus toward a cell line. A value of -1 indicates the therapeutic virus has 10 times less killing activity than wild-type virus toward a cell line.

Animal Experiments. All animal methods and procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee (IACUC). CWR22rv mouse tumors were established by injecting 4×10^6 cells s.c. in the flanks of athymic nude mice (6 weeks old, males). The injected mice were castrated 3 days after cellular injection. Mice with similar tumor sizes were randomized 3 weeks after injection (nine tumors from six mice for each group) and treated with 2×10^7 IFU of either AdE4PSESE1a or AdCMV-EGFP (a replication-deficient adenovirus used as a negative control) in 100 μ L 1× PBS via intratumoral injections. In addition, seven tumors from six mice were treated with 5×10^7 IFU of AdE4PSESE1a in 50 μ L 1× PBS via tail vein injection. Tumor appearance and tumor size were measured once every week with calipers, and the following formula was applied to calculate tumor volume: length \times width² \times 0.5236 (23). Tumor growth curves were drawn according to the weekly measurement. Data are expressed as fold-increase in tumor size, obtained by assessing tumor size relative to the initial size at the time of virus or vehicle injection. Significant differences between treatment and control groups were analyzed using Student's *t* test.

Fluorescent Imaging. We used a Berthold LB981 NightOwl System (EG&G Berthold, Bad Wildbad, Germany) to monitor the expression of EGFP in AdE4PSESE1a- or AdCMV-GFP-treated tumors. The Berthold LB981 NightOwl System is an optical imager used for the measurement of or near-IR emitting molecules. It contains a Peltier cooled backlit CCD camera (576 \times 385 pixels) housed within a light-tight enclosure. The excitation source is filtered using an HQ 470 bandpass filter (Chroma Technology Corp., Rockingham, VT) and uniformly illuminates the field of view of the mouse. The emission spectrum is filtered using an HQ 525 bandpass filter (Chroma Technology) to enhance the GFP fluorescence relative to the autofluorescence signal from endogenous tissue. The fluorescent images of the tumor were taken immediately and on days 3, 7, 14, 21, and 28 post-viral injections. The mice were sedated (1.5 mg/kg acepromazine and torbugesic by i.m. injection), positioned in the light-tight

chamber, and imaged with an exposure time of 100 ms. The change in fluorescent signal was depicted on a graph.

Histology and Immunohistochemistry. Tumors were removed, immediately fixed in formalin, and embedded in paraffin. Four-micrometer sections were cut into histologic sections, stained with H&E, and examined under a light microscope. For immunohistochemistry, tumor sections were deparaffinized using a sequential protocol of xylene and hydrated with graded ethanol and distilled water. All markers were determined after antigen retrieval (sample boiled for 4 minutes in a 0.01 mol/L sodium citrate buffer in a microwave oven and cooled to room temperature). After rinsing with distilled water, slides were immersed in 3% hydrogen peroxide for 20 minutes at room temperature to quench endogenous peroxidase activity. The slides were rinsed with distilled water, washed twice with PBS for 3 minutes, and blocked with superblock (Scytek Laboratories, Burlingame, CA) in a humidified chamber for 60 minutes at room temperature. After rinsing with PBS, the slides were blocked with avidin from an avidin-biotin kit (Vector Laboratories, Inc., Burlingame, CA) for 15 minutes, washed with PBS, and blocked with biotin in a humidified chamber for 15 minutes at room temperature. A rabbit polyclonal antibody to adenovirus 5 (Abcam, Cambridge, MA) was applied to slides at a dilution of 1:200. Normal rabbit immunoglobulin G was used as a control. The slides were reacted with primary antibodies overnight in humidified chambers at 4°C. After being rinsed once with PBS, a biotinylated polyclonal anti-rabbit antibody was applied to slides at a dilution of 1:500 and incubated for 1 hour. After washing with PBS, slides were incubated with avidin-biotin-peroxidase complex (Vector Laboratories) for 1 hour, washed once with PBS, stained with freshly prepared 3,3'-diaminobenzidine solution for 15 minutes, and counterstained with hematoxylin.

In situ Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay. The *in situ* apoptosis detection kit was purchased from Roche Diagnostics (Indianapolis, IN). Tumor tissue sections were deparaffinized using a sequential xylene protocol and rehydrated through gradients of ethanol and distilled water. Slides were treated with 10 mmol/L Tris solution containing 1 µg/mL proteinase K for 15 minutes. All slides were rinsed thrice with PBS and incubated with 100 µL terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction mixture (or 100 µL control labeling solution for negative control) in a humid chamber at 37°C for 30 minutes. The slides were washed thrice with PBS and incubated with 100 µL TUNEL POD solution in a humid chamber at 37°C for 30 minutes. After washing with PBS, the slides were stained with freshly prepared 3,3'-diaminobenzidine solution for 10 minutes, rinsed with PBS, and counterstained with hematoxylin.

Results

PSES Restricted the Expression of E4 Gene in HEK293 and HER911, Thus Controlling Adenoviral Replication. To control adenoviral replication in a prostate-specific manner, we developed a new strategy to produce a TRRA. This new strategy, illustrated in Fig. 1A, places the adenoviral *E1a* gene at the right end of the adenoviral genome to avoid potential interference from the adenoviral packaging signal and places the prostate-specific enhancer, PSES, between *E1a* and *E4* genes to direct their expression. A CMV promoter-controlled EGFP expression cassette is cloned at the left end of the adenoviral genome to allow the monitoring of viral propagation *in vitro* and virus distribution *in vivo*. The viral genome was released from the cloning vector by restriction enzyme *PacI* digestion and transfected into helper cells, HEK293, HER911, and HER911E4. Green fluorescent cells could be seen under the inverted fluorescent microscope on the day following transfection in all three cell lines (data not shown). For HER911E4, which expresses adenoviral *E1a* and *E4* proteins, the number of green fluorescent cells increased with time and formed comet-like plaques 7 days after transfection; at the same time, cytopathic effect could be detected under a light microscope. For

HEK293 and HER911 helper cells, which only express adenoviral *E1* proteins, the quantity and brightness of green fluorescent cells did not change significantly with time (Fig. 1B). Under a light microscope, the appearance of cells was normal and no cytopathic effect could be detected up to 7 days after transfection. This result showed that *E4* was under the tight control of PSES enhancer, which was not active in HEK293 and HER911 cells.

The gene structure of AdE4PSESE1a produced in HER911E4 was confirmed by PCR using genome DNA extracted from AdE4PSESE1a-infected cells. As shown in Fig. 2, use of *E4* reverse (primer 1) and PSES reverse (primer 3) primers amplified an expected DNA fragment size of 545 bp, whereas *E4* reverse (primer 1) and PSES forward (primer 2) primers did not produce a PCR product because they face the same direction. In addition, a combination of PSES forward (primer 2) and *E1a* reverse (primer 4) primers produced an expected DNA fragment size of 699 bp, whereas PSES reverse (primer 3) and *E1a* reverse (primer 4) did not produce a PCR product because they face the same direction. The above results showed that no gross rearrangement of the inserted gene occurred during virus production. AdE4PSESE1a was then amplified in HER911E4, purified by CsCl gradient method, and titered (see Materials and Methods).

Differential Adenoviral Infection Susceptibility. Because of every cell line expressing different amounts of Coxsackie-adenovirus receptor for adenovirus, their adenoviral infectivities were expected to vary. Therefore, it is necessary to establish individual infection conditions for each cell line to achieve similar infectivity among cell lines (24). We conducted an experiment to normalize the susceptibility of tumor cell lines to adenoviral infection using AdCMV-Luc, an *E1/E3*-deleted replication-deficient adenovirus that carries a luciferase reporter gene under the control of CMV promoter. Figure 3A illustrates the various viral doses required for similar infectivity in C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, MCF10A, and HEK293. For example, C4-2 was infected with 20 virus particles per cell to test whether the different viral doses shown in Fig. 3A for each cell result in similar luciferase activity. The cells indicated above were infected with AdCMV-Luc for 48 hours, followed by luciferase assay. As shown in Fig. 3B, a similar luciferase activity (about 10⁵ luciferase units) could be obtained among cell lines when adenoviral

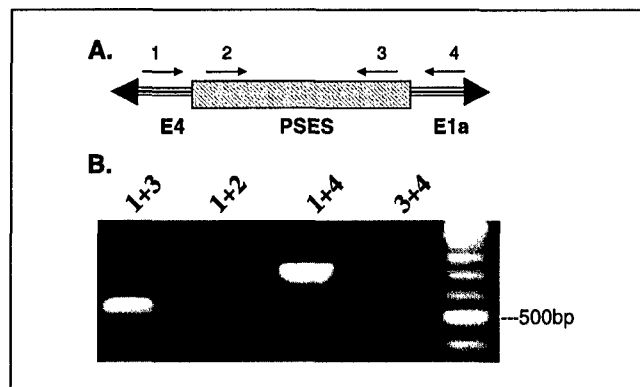


Figure 2. Confirmation of the gene structure of AdE4PSESE1a. The viral genome DNA produced in AdE4PSESE1a-infected HER911E4 was extracted by the phenol/chloroform method and used as a template for PCR. A, E4-PSES-E1a expression cassette in the right end of AdE4PSESE1a. Arrows, selected primers; E4-reverse (1), PSES-forward (2), PSES-reverse (3), and E1a-reverse (4). B, PCR results with the different primer pairs.

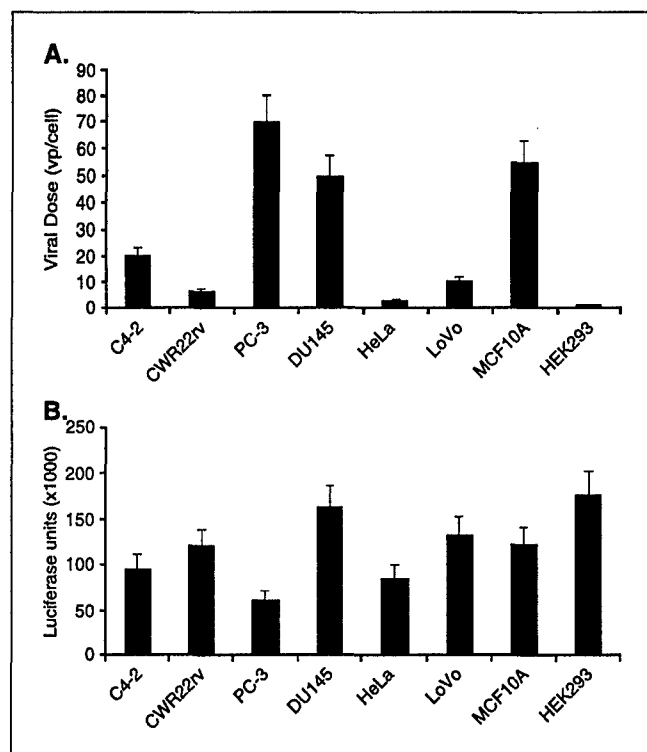


Figure 3. Standardization of infectivity in various cell lines. C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, MCF10A, and HEK293 cells were seeded (2.5×10^5 cells per well) in a 12-well plate 1 day before viral infection. Cells were infected with serial doses of AdCMV-Luc from 0.05 to 37.25 multiplicity of infection. The media were changed 24 hours after addition of the virus. The cells were harvested 48 hours after infection for luciferase assay. **A**, different viral doses required for similar infectivity in all cell lines. **B**, similar luciferase activity was obtained among cell lines when adenoviral infectivities were standardized.

infectivities were standardized. This result was applied to all subsequent *in vitro* experiments.

AdE4PSESE1a Directed the Expression of E1a and E4 in PSA/PSMA-Positive Prostate Cancer Cells. To test whether PSES enhancer could control E1a protein expression in PSA/PSMA-positive prostate cancer cells, we infected C4-2 and CWR22rv (PSA/PSMA-positive, androgen-independent prostate cancer cells) and PC-3, DU145, HeLa, LoVo, and MCF10A (PSA/PSMA-negative cells) with either AdE4PSESE1a or Ad-wt. Owing to adenoviral replication efficiency varying with each cell line, Ad-wt was used as a control for similar infectivities. Figure 4A depicts the ability of Ad-wt to direct E1a protein expression in all seven cell lines tested. On the other hand, AdE4PSESE1a directed E1a protein expression only in PSA/PSMA-positive prostate cancer cells, C4-2, and CWR22rv. E1a protein expression in PSA/PSMA-negative cells was undetectable or very low. Due to the lack of accessibility to E4-specific antibodies, reverse transcription-PCR was done to compare the expression profiles of E4 mRNA among several human prostate cancer cells and nonprostatic cancerous and normal cells. Figure 4B shows that AdE4PSESE1a expressed high amounts of E4 mRNA only in PSA/PSMA-positive prostate cancer CWR22rv and C4-2, but not in PSA/PSMA-negative PC-3, DU145, HeLa, LoVo, and MCF10A. These results not only showed that PSES retained its prostate specificity in AdE4PSESE1a but also indicated that a single enhancer core could be used to control the expression of two groups of viral genes in a bidirectional manner.

AdE4PSESE1a Propagated Selectively in PSA/PSMA-Positive Prostate Cancer Cells but Not in PSA/PSMA-Negative Cancer Cells. We did an *in vitro* viral replication assay to compare the viral replication efficiency of AdE4PSESE1a and Ad-wt. This would also determine whether AdE4PSESE1a could replicate selectively in the PSA/PSMA-positive prostate cancer cells. Cells were infected with AdE4PSESE1a or Ad-wt and the media were changed 24 hours post viral infection. The supernatants were harvested for titer assay 2 days after the medium change. Viral titer for AdE4PSESE1a was normalized by one for Ad-wt and values were represented as \log_{10} phase. As shown in Table 1, AdE4PSESE1a propagated as efficiently as Ad-wt in PSA/PSMA-positive C4-2 and CWR22rv. On the other hand, AdE4PSESE1a showed a limited replication activity in PSA/PSMA-negative cells. Compared to Ad-wt, it produced 100-fold fewer viruses in LoVo, 500-fold fewer viruses in DU145, HeLa, and HEK293, 1,000-fold fewer viruses in PC-3, and 5,000-fold fewer viruses in MCF10A cells.

We conducted another experiment to investigate the tissue-restricted replication of AdE4PSESE1a. PSA/PSMA-positive and PSA/PSMA-negative cells were infected with AdE4PSESE1a and monitored daily under a fluorescent microscope up to 5 days. EGFP expression represented AdE4PSESE1a infection. At 1 day after infection, EGFP-expressing cells were easily detected in all cell types tested (Fig. 5). The number and intensity of green cells increased in PSA/PSMA-positive prostate cancer cells but not in PSA/PSMA-negative cells. At day 5, a comet-like patch of green cells had formed only in PSA/PSMA-positive prostate cancer cells. At the same time, we could detect virus plaques under a light

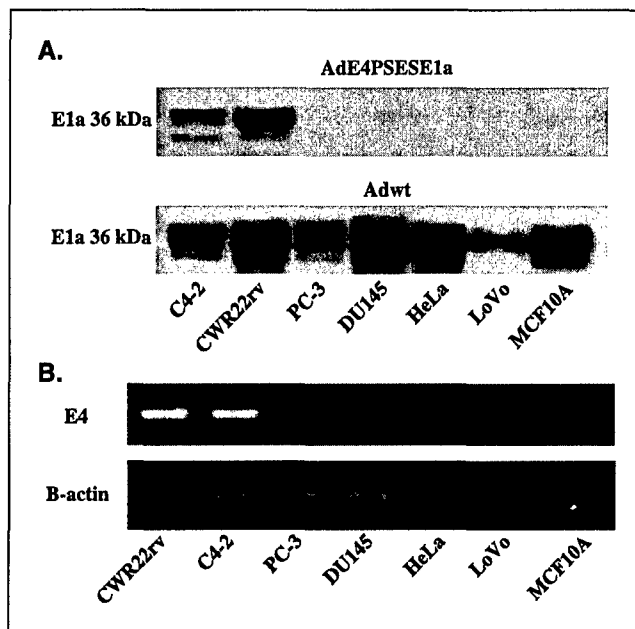


Figure 4. AdE4PSESE1a-directed E1a and E4 expression in human prostate cancer cell lines. **A**, E1a protein expression. C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, and MCF10A cells were infected with standardized doses of either AdE4PSESE1a or Ad-wt and harvested for protein preparation 24 hours postinfection. E1a protein was detected by Western blot and probed with a polyclonal antibody to Ad5 E1a protein. Ad-wt was used as a control. **B**, E4 mRNA accumulation. C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, and MCF10A cells were infected with AdE4PSESE1a and total RNA was prepared for reverse transcription-PCR 24 hours postinfection. β -Actin was used as an internal standard of RNA loading in each sample.

Table 1. Tissue/tumor-specific replication ability of AdE4PSESE1a

Cell lines	Input doses* (IFU)	Output viral doses (LD ₅₀ [†])		
		AdE4	Adwt	AdE4/Adwt
C4-2	6.6×10^4	10^6	10^6	1
CWR22rv	2×10^4	10^6	10^6	1
PC-3	2.3×10^5	10^2	10^5	10^{-3}
DU145	1.6×10^5	5×10^2	10^5	5×10^{-3}
HeLa	8×10^3	5×10^3	10^6	5×10^{-3}
LoVo	3.3×10^4	10^4	10^6	10^{-2}
MCF10A	1.8×10^5	5×10^2	10^6	5×10^{-4}
HEK293	3.3×10^3	5×10^2	10^5	5×10^{-3}

Cells were seeded and infected with AdE4PSESE1a, and the supernatants were harvested for titer assay as described in Materials and Methods.

*Input viral doses mean the virus doses used to infect cells.

[†]Output viral doses mean the titered virus doses in titer assay.

[‡]The virus production was expressed as a LD₅₀ value (the dilution factor that caused a cytopathic effect in at least 4 wells of cells in a row on a 96-well plate on day 7).

microscope. All these results showed that the replication of AdE4PSESE1a is tightly controlled by PSES and restricted to PSA/PSMA-positive cells.

AdE4PSESE1a Showed Specific Cell Killing Ability in PSA/PSMA-Positive Cancer Cells. To test the tissue/tumor-specific killing activity of AdE4PSESE1a, serial dilutions of AdE4PSESE1a and Ad-wt were applied to C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, A549, MCF10A, and HEK293 in 96-well plates. Cells were monitored under the microscope daily. Figure 6 presents the viral doses that cause a cytopathic effect in at least four wells of cells in a line of 96-well plates used for each dose. AdE4PSESE1a was able to kill C4-2 and CWR22rv at the same doses as Ad-wt. On the other hand, AdE4PSESE1a required 100-fold more viruses than Ad-wt to kill LoVo, 500-fold more viruses to kill DU145 and HeLa, 1,000-fold more viruses to kill PC-3, and MCF10A, and 10,000-fold more viruses to kill HEK293 cells. This result indicates that the killing activity for AdE4PSESE1a was the same as for Ad-wt in PSA/PSMA-positive cancer cells and significantly attenuated in PSA/PSMA-negative prostate cancer cells and nonprostatic cancer cells.

AdE4PSESE1a Was Effective against the Growth of Androgen-Independent CWR22rv Prostate Tumors. CWR22rv s.c. tumors were established in athymic nude mice as described in Materials and Methods. The mice were castrated 3 days after CWR22rv inoculation to test whether AdE4PSESE1a was able to eliminate androgen-independent tumors in a castrated host. Mice were randomized 3 weeks after cell inoculation (nine tumors from six mice in each group) and received intratumoral injections of 2×10^7 IFU AdE4PSESE1a or AdCMV-GFP. A replication deficient adenovirus was used as a negative control. In addition, seven tumors from six mice were treated i.v. with 5×10^7 IFU of AdE4PSESE1a in 50 μ L 1 \times PBS at 1-week intervals. Tumor sizes were monitored once a week. Figure 7A shows that tumor growth was significantly retarded in the AdE4PSESE1a-treated groups regardless of the route of viral injection compared with those in the AdCMV-GFP-treated group. Two tumors from two animals disappeared in the AdE4PSESE1a intratumoral injection group (Fig. 7A). Three tumors from three different animals disappeared in the tail-vein injection group (Fig. 7C). When tumors from the intratumoral injection group

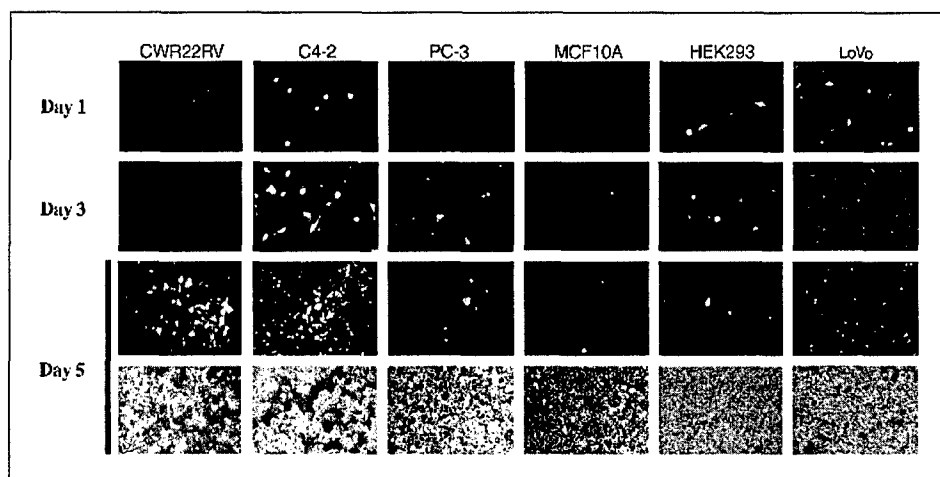


Figure 5. Tissue/tumor-specific replication ability of AdE4PSESE1a. CWR22rv, C4-2, PC3, MCF10A, and HEK293 cells were infected with standardized doses of AdE4PSESE1a and monitored daily under fluorescent microscopy and light microscopy up to 5 days after viral infection. EGFP expression represented AdE4PSESE1a infection ($\times 20$).

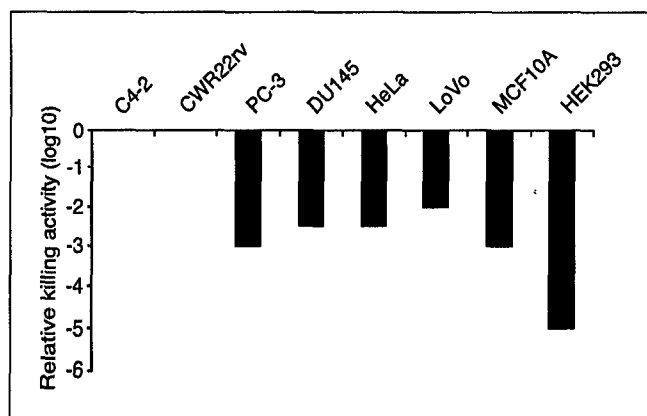


Figure 6. Tissue/tumor-specific killing activity of AdE4PSESE1a. Serial dilutions of AdE4PSESE1a and Ad-wt were applied to C4-2, CWR22rv, PC-3, DU145, HeLa, LoVo, MCF10A, and HEK293 in 96-well plates. The virus doses are the same in the first row. Cells were monitored under the microscope daily. The viral killing activity was shown as an LD₅₀ value. A tissue/tumor-specific killing index was obtained by dividing the LD₅₀ value of therapeutic viruses to that of wild-type. The value is expressed as a log₁₀ scale, such that a value of 0 indicates the therapeutic virus has the same killing activity as wild-type virus toward a cell line. A value of -1 indicates the therapeutic virus has 10 times less killing activity than wild-type virus toward a cell line.

were harvested, we observed that AdCMV-GFP- and AdE4PSESE1a-treated tumors exhibited different appearances. In the AdCMV-GFP-treated group, the tumors were big, solid, and evenly hard. On the other hand, in the AdE4PSESE1a-treated group, tumors were small with some fragmentary and necrotic cotton-shaped tissues embedded in a turbid liquid. Tumor histology revealed that small, patchy island-shaped tumor tissues were surrounded by extensive necrotic tissue inside the tumors in the AdE4PSESE1a-treated group (Fig. 8A and C). However, the periphery of the necrotic area consisted of a shallow layer of healthy tumor cells (Fig. 8C), suggesting that viruses did not reach the outer rim of the tumors, which were still growing actively. This phenomenon might contribute to a minor gain in tumor size in the very late phase of the animal experiments, especially in tumors treated by intratumoral injection (Fig. 7A). In the AdCMV-GFP-treated group, the tumor cells were healthy and evenly distributed inside the tumors with very little necrotic tissue (Fig. 8B and D). Anti-adenovirus-5 immunohistochemical staining revealed that extensive viral infection existed throughout the treated tumors, mainly in tumor cells at the border between tumor and necrosis in AdE4PSESE1a-treated tumors (Fig. 8E). Anti-adenovirus-5 immunohistochemical staining was absent in AdCMV-GFP-treated tumors (Fig. 8E).

In situ TUNEL assays were done to detect apoptotic bodies in the AdE4PSESE1a-treated tumors. Dark-brown nuclear-staining cells were found around the border between the tumor necrosis and the tumor (Fig. 8G), indicating that programmed cell death is involved in the process of tumor killing. No dark-brown cells could be found in either the tumor or necrotic areas (Fig. 8H).

Infection of AdE4PSESE1a Could Be Monitored via a Fluorescent Imaging System. AdE4PSESE1a carries EGFP under the control of CMV promoter to track its infection and distribution in animal tumors via a fluorescent imaging system. The expression of EGFP in AdE4PSESE1a-treated tumors was imaged with an LB981 Molecular Light Imager (Night OWL) system (25) at 3 to 28 days after viral injection. The EGFP signal was negative right

after AdE4PSESE1a injection because the adenovirus did not express enough EGFP at that time (data not shown). AdCMV-GFP-treated tumors never showed an EGFP signal compared with background. A strong signal showed up on the images 3 days after AdE4PSESE1a injection (Fig. 9A), which suggests that the virus infected the tumor cells and replicated rapidly. During the following 2 weeks, fluorescence dropped slightly. Three AdE4PSESE1a-treated (intratumoral injection) tumors showed consistent, significant EGFP signals throughout the treatment period. The data for these three tumors are shown in Fig. 9B.

Discussion

TRRA provides an efficient *in vivo* gene delivery method that overcomes the problems encountered in the majority of gene

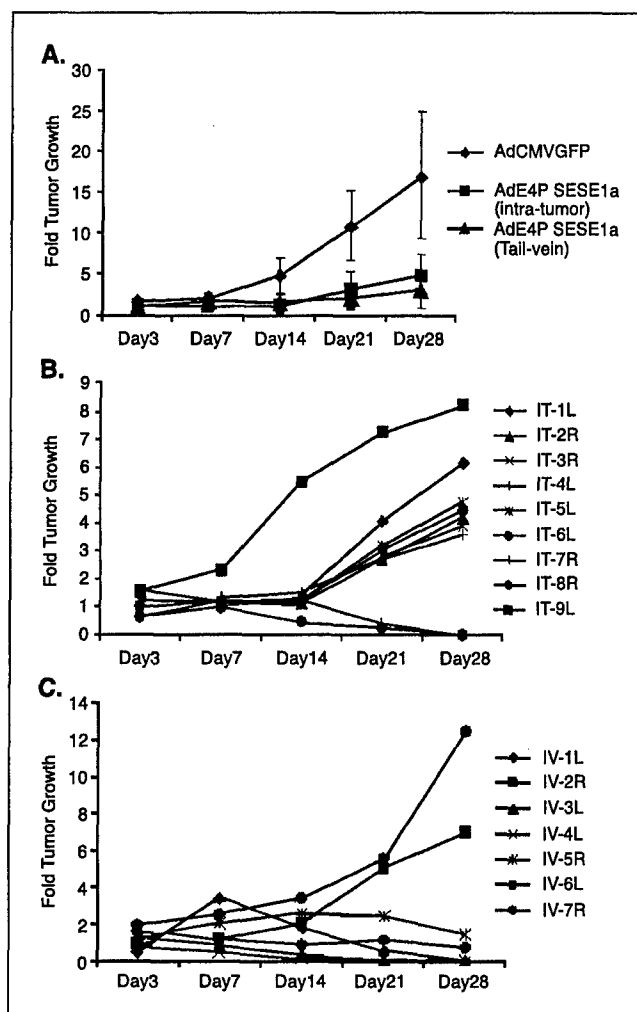


Figure 7. Evaluation of antitumor effect of AdE4PSESE1a *in vivo*. A CWR22rv prostate tumor s.c. model was established in athymic nude mice. Mice were randomized 3 weeks after cell inoculation (nine tumors from six mice in each group) and treated with 2×10^7 IFU of either AdE4PSESE1a or AdCMV-EGFP in 100 μ L $1 \times$ PBS via intratumoral injections. In addition, seven tumors from six mice were treated i.v. with 5×10^7 IFU of AdE4PSESE1a in 50 μ L $1 \times$ PBS at 1-week intervals. Tumor sizes were monitored once every week. A, tumor size from each group was averaged. B, the sizes of tumors treated by intratumoral injection were plotted individually. Two tumors from two mice disappeared in the AdE4PSESE1a intratumoral injection group. C, the sizes of tumors treated by i.v. injection were plotted individually. Three tumors from three mice disappeared in the tail-vein injection group.

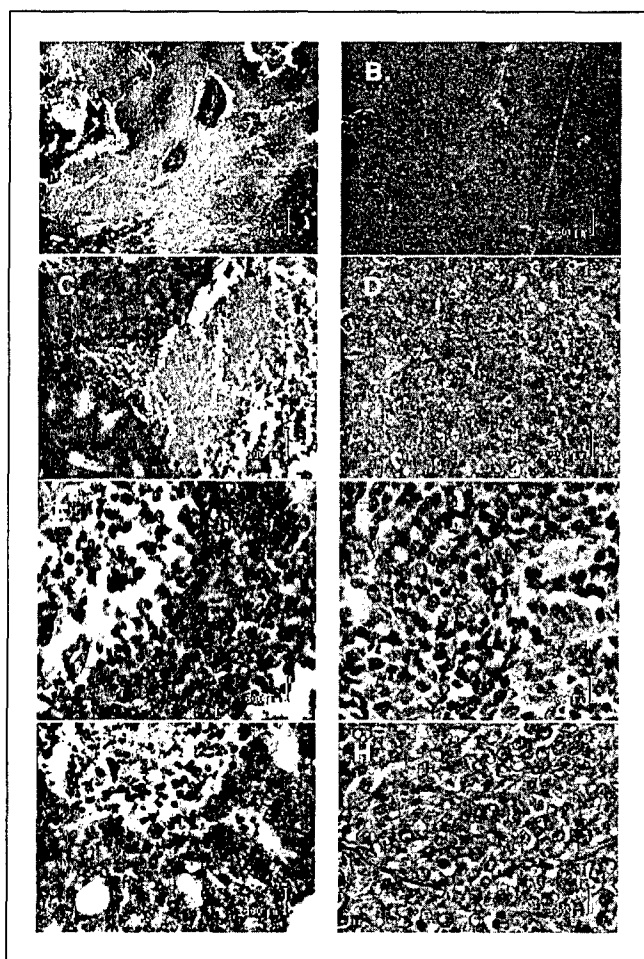


Figure 8. Histologic representations of virus-treated tumors. At 8 weeks after virus injection, tumor samples were collected for paraffin sections followed by H&E staining. Small, patchy, island-shaped tumor tissues were surrounded by extensive non-cell necrotic tissue inside the tumors in the AdE4PSESE1a-treated group (A, H&E $\times 4$; C, H&E $\times 10$). In the AdCMV-GFP-treated sections, very little necrotic tissue could be seen (B, H&E $\times 4$; D, H&E $\times 10$). Anti-adenovirus-5 immunohistochemical staining revealed that extensive viral infection exists throughout the treated tumors, mainly in tumor cells at the border between tumor and necrosis (E, $\times 20$). Anti-adenovirus-5 immunohistochemical staining was absent in nontreated tumors (F, $\times 20$). *In situ* TUNEL assays were done to detect apoptotic bodies in the AdE4PSESE1a-treated tumor group. Dark-brown nuclear-staining cells were found bordering the tumor necrosis and the tumor (G, $\times 20$) but no dark-brown cells could be found either in the tumor or in necrosis areas (H, $\times 20$).

therapy protocols. One way to construct a TRRA is by using tissue/tumor-specific promoters to control the expression of viral regulatory proteins (26, 27). There are six early transcription units in the adenovirus backbone. The first unit to be activated after entry into the nucleus is the *E1a/E1b* region. The adenovirus *E1a* proteins play important roles in the process of adenovirus gene expression and transcription (28, 29). Tissue- or tumor-specific promoters/enhancers are used to replace the *E1a* promoter-enhancer region, with the rationale that expression of *E1a*, and therefore of the whole adenovirus transcription program, will depend on these tissue- or tumor-specific promoters (30). This is the most commonly used strategy to make a TRRA. Currently, four promoters, including kallikrein 2, PSA, rat probasin, and osteocalcin, are under extensive investigation for producing prostate-restricted replicative adenovirus (2, 5, 7, 8). In our previous investigations, PSES showed high activity specifically in C4-2 and

CWR22rv PSA/PSMA-positive and androgen-independent prostate cancer cells (12). A replication-deficient recombinant adenovirus carrying the luciferase reporter gene under the control of PSES enhancer drove high luciferase activity almost exclusively in PSA/PSMA-positive prostate cancer cells regardless of androgen status. We believe that PSES has several advantages over kallikrein 2 (7), PSA (2), rat probasin (31, 32), and osteocalcin promoters (3, 4). First, PSA, promoters from kallikrein 2, and rat probasin are highly androgen-dependent and may not be the best choice for patients undergoing androgen-ablation therapy. Second, probasin is a murine PSA, and the tissue-specific activity of its promoter in humans has not been extensively tested. Third, although our study of osteocalcin promoter indicates that osteocalcin promoter is active in androgen-independent cancer cells, our data also suggest that osteocalcin promoter may be active in several other organs besides bone and requires more vigorous testing to clarify its tissue specificity (33).

Early studies of *E1a*-based replicative-competent adenoviruses showed various degrees of success as well as certain limitations. The success of TRRA depends on the tightness of the tissue/tumor-restricted promoter/enhancer. Some promoters are highly influenced by the context of the vector backbone, resulting in leaky *E1a* expression and loss of specificity. Combinatorial control of Ad5 *E1a* and *E1b*, for example *E1a* under the control of probasin promoter and *E1b* under the control of PSA promoter, was shown to achieve better tissue-specific replication than control of *E1* gene expression alone (7, 8). However, it may not be easy to find two tightly controlled tissue-specific promoters for any single tumor type, such as breast cancer. Besides, juxtaposing promoters with heterogenous sequences could result in promoter competition, squelching of transcription factors, or loss of tissue specificity (34). On the other hand, juxtaposing promoters with a homologous region could result in homologous recombination and deletion of transgenes important for viral expression. Using a single promoter to drive both *E1a* and *E1b* genes could avoid homologous recombination, promoter competition, and the squelching effect of transcription factors during gene transcription (35). However, we also experienced leaky replication with this approach depending on the promoter as well as the cell line.

Besides *E1a* and *E1b* genes, the *E4* genes also play critical roles in efficient viral replication and can be controlled by a tissue/tumor-specific promoter. The adenovirus *E4* gene constitutes around 10% of the viral genome and is located at the right end of the viral genome. It encodes several regulatory proteins with pleiotropic functions. Genetic analysis has shown that *E4* products are essential for productive virus infection. Removal of the *E4* region severely disrupts viral gene expression in transduced cells and shows that *E4* products play vital roles in viral infection. The *E4* proteins are involved in several levels of regulation of cellular and viral gene expression, viral DNA replication, late viral mRNA splicing and accumulation, viral protein synthesis, host shutoff, virus assembly, *E2* expression, and adeno-associated virus helper function (36–39). Several sets of differentially spliced mRNAs are generated from the *E4* region during viral infection (40–43).

However, new strategies to selectively control *E4* gene expression via promoter/enhancer are still uncommon. The reason may be that *E4* proteins are cytotoxic (44). However, *E1a* and *E4* targeting may synergize each other, resulting in both a highly selective TRRA and neutralization of *E4* protein

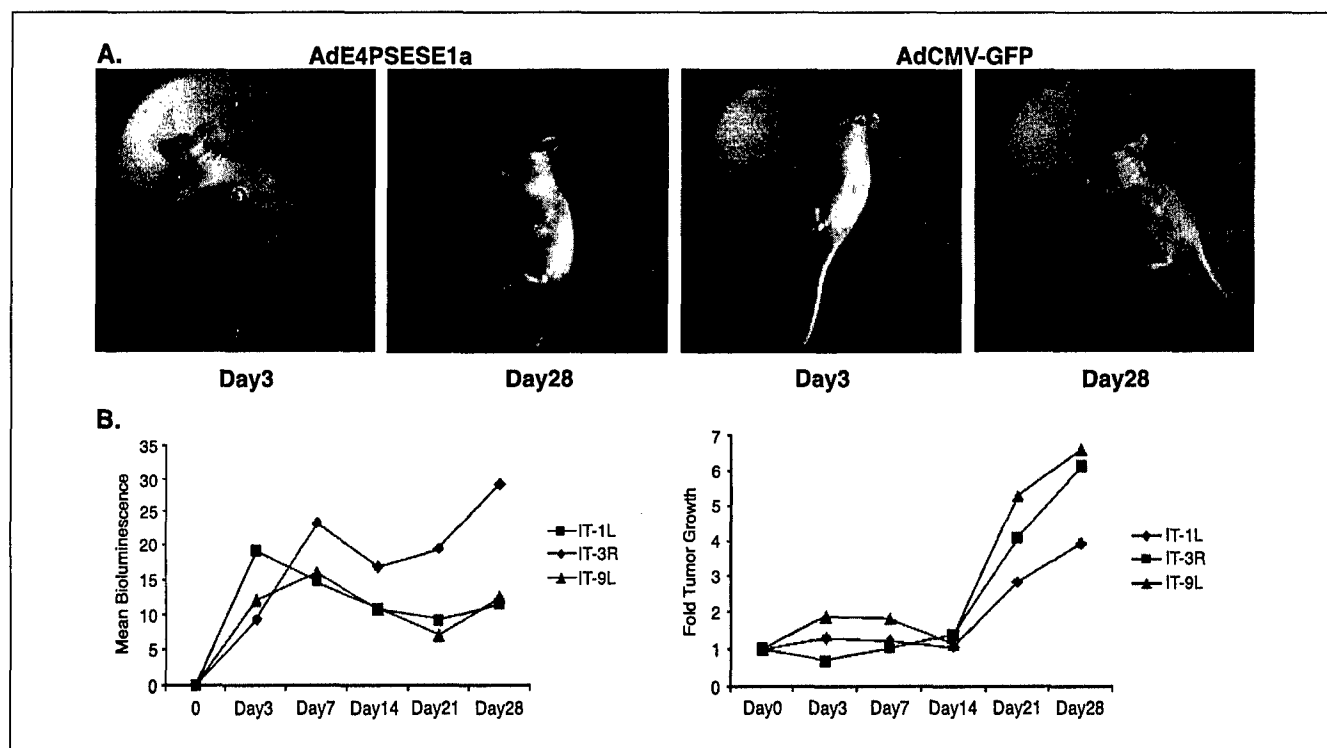


Figure 9. GFP bioluminescence and tumor size in s.c. tumor models. The LB981 Molecular Light Imager (Night OWL) system can track virus infection and distribution in animal tumor models because AdE4PSESE1a carries an EGFP. AdCMV-GFP was used as a control. **A**, bioluminescence in AdE4PSESE1a-treated or AdCMV-GFP-treated tumors. **B**, three AdE4PSESE1a-treated (intratumoral injection) tumors showed consistent, statistically significant EGFP signals throughout the treatment period.

cytotoxicity. Therefore, controlling both *E1a* and *E4* regions by a single promoter can result in a more specific viral replication. There are several reports of successfully controlling adenoviral replication by controlling the expression of *E1a* and *E4* genes (10, 11, 30, 45). Some studies controlling adenovirus *E1a* and *E4* genes by tissue/tumor-restricted promoters have obtained high tissue/tumor-specific targeting (11, 45, 46). For example, the adenovirus mutant ONYX-411, in which the *E1a* and *E4* genes were driven by different copies of the human E2F promoter, showed a high selectivity in retinoblastoma-deficient tumor cells (11). OVA002 has the *E1a* and *E4* genes under the control of E2F promoter and human telomerase promoter, respectively, and shows higher tumor selectivity than OVA001, in which *E1a* is driven by the E2F promoter and *E4* is under its own promoter (45).

Previously we showed that PSES exhibits very restricted tissue-specific activity in PSA/PSMA-positive prostate cancer cells. In this report, we constructed a TRRA AdE4PSESE1a by inserting *E1a* in the right end of adenoviral genome to avoid potential interference from the adenoviral packaging signal and putting PSES between *E1a* and *E4* genes to control the expression of *E1a* and *E4* genes simultaneously. AdE4PSESE1a showed tightly controlled replication. It cannot replicate well in all nonprostatic cancer cells tested thus far, not even in HEK293 and HER911, which express *E1a* and *E1b* proteins. AdE4PSESE1a replicated as efficiently as Ad-wt in PSA/PSMA-positive cancer cells. The diminishment of AdE4PSESE1a to replicate in HEK293 and HER911 indicates the stringent requirement of *E4* proteins for efficient viral replication. We detected low levels of *E4* mRNA expression in PC-3 and DU145.

However, AdE4PSESE1a produced 1,000-fold fewer viruses than Ad-wt in PC-3, suggesting that adenovirus requires more *E4* protein expression for efficient viral replication.

AdE4PSESE1a showed better tumor suppression activity for androgen-independent prostate tumors in castrated hosts than AdCMV-GFP. In particular, two tumors disappeared in the intratumoral injection group and three tumors disappeared in the tail-vein injection group. AdE4PSESE1a has another advantage over other TRRAs. It encodes a reporter, EGFP, which allows us to track the virus infection, replication, and cell killing *in vitro* continuously with a fluorescent microscope. EGFP also allows us to conduct live imaging to monitor viral infection, amplification, and distribution continuously through a CCD camera, such as the Berthold LB981 NightOwl System used in this study. Figure 9A depicts a dramatic increase in EGFP expression 3 days after virus injection. We believe this is due to rapid viral replication and spreading of AdE4PSESE1a, owing to AdCMV-GFP-treated tumors not exhibiting the same phenomenon. It is quite surprising that rapid viral amplification only occurred in the first few days (Fig. 9A) and EGFP signals started to drop 5 days after virus injection. It is not clear what reduced viral replication after the first phase of acute replication. It seems that tumor growth and death rate reached a balance in the first 2 weeks after virus injection and then tumor growth rate exceeded death rate, resulting in new tumor growth (Fig. 9B). The regrowth of tumor restimulated viral replication, as indicated by a slight rebound of the EGFP signal 21 days after viral injection (Fig. 9B). The immunohistochemistry indicated persistent viral replication (Fig. 8E) for 28 days after virus injection. This result is consistent with Harrison's report that

complete tumor responses to adenovirus dl309 therapy are rarely achieved despite viral persistence in the tumor (47). For a TRRA to succeed in the clinic, it is critical to understand how viral replication is slowed down after the initial acute replication phase.

In conclusion, we developed a prostate-restricted replicative adenovirus, AdE4PSESE1a, by controlling the expression of *E1a* and *E4* genes with a chimeric prostate-specific transcriptional enhancer, PSES. AdE4PSESE1a showed similar killing and replication activities to Ad-wt in PSA/PSMA-positive androgen-independent human prostate cancer cells, and much lower activities in PSA/PSMA-negative prostate cancer cells and nonprostate cancer cells. The inhibition of the growth of androgen-independent CWR22rv s.c. tumors in castrated animals indicated the therapeutic potential of AdE4PSESE1a for the treatment of androgen-independent cancers. This study revealed that adenoviral replication slowed down after several days of

acute replication, a phenomenon that needs to be overcome to optimize the therapeutic use of TRRA. We believe that AdE4PSESE1a could be further improved by replacing the CMV-EGFP expression cassette with a therapeutic gene, such as an apoptosis inducer or a suicide gene controlled by a tissue specific promoter, to further enhance its therapeutic efficacy. This report also provides a new strategy to construct a TRRA for those tumor types that have limited tissue/tumor-specific promoters available.

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References

- Huggins C, Hodges CV. Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *J Urol* 2002; 168:9-12.
- Gotoh A, Ko SC, Shirakawa T, et al. Development of prostate-specific antigen promoter-based gene therapy for androgen-independent human prostate cancer. *J Urol* 1998;160:220-9.
- Koenenman KS, Kao C, Ko SC, et al. Osteocalcin-directed gene therapy for prostate-cancer bone metastasis. *World J Urol* 2000;18:102-10.
- Ko SC, Cheon J, Kao C, et al. Osteocalcin promoter-based toxic gene therapy for the treatment of osteosarcoma in experimental models. *Cancer Res* 1996;56:4614-9.
- Matsubara S, Wada Y, Gardner TA, et al. A conditional replication-competent adenoviral vector, Ad-OC-E1a, to cotarget prostate cancer and bone stroma in an experimental model of androgen-independent prostate cancer bone metastasis. *Cancer Res* 2001;61: 6012-9.
- Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. *Cancer Res* 1997;57:2559-63.
- Yu DC, Sakamoto GT, Henderson DR. Identification of the transcriptional regulatory sequences of human kallikrein 2 and their use in the construction of calydon virus 764, an attenuated replication competent adenovirus for prostate cancer therapy. *Cancer Res* 1999;59:1498-504.
- Yu DC, Chen Y, Seng M, Dilley J, Henderson DR. The addition of adenovirus type 5 region E3 enables calydon virus 787 to eliminate distant prostate tumor xenografts. *Cancer Res* 1999;59:4200-3.
- Hsieh CL, Yang L, Miao L, et al. A novel targeting modality to enhance adenoviral replication by vitamin D(3) in androgen-independent human prostate cancer cells and tumors. *Cancer Res* 2002;62: 3084-92.
- Banerjee NS, Rivera AA, Wang M, et al. Analyses of melanoma-targeted oncolytic adenoviruses with tyrosinase enhancer/promoter-driven E1A, E4, or both in submerged cells and organotypic cultures. *Mol Cancer Ther* 2004;3:437-49.
- Johnson L, Shen A, Boyle L, et al. Selectively replicating adenoviruses targeting deregulated E2F activity are potent, systemic antitumor agents. *Cancer Cell* 2002;1:325-37.
- Lee SJ, Kim HS, Yu R, et al. Novel prostate-specific promoter derived from PSA and PSMA enhancers. *Mol Ther* 2002;6:415-21.
- Graham FL. Growth of 293 cells in suspension culture. *J Gen Virol* 1987;68:937-40.
- Fallaux FJ, Kranenburg O, Cramer SJ, et al. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum Gene Ther* 1996;7:215-22.
- He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses. *Proc Natl Acad Sci U S A* 1998;95:2509-14.
- Thalmann GN, Anezinis PE, Chang SM, et al. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer Res* 1994;54:2577-81.
- Tepper CG, Boucher DL, Ryan PE, et al. Characterization of a novel androgen receptor mutation in a relapsed CWR22 prostate cancer xenograft and cell line. *Cancer Res* 2002;62:6606-14.
- Kaighn ME, Narayan KS, Ohnuki Y, Lechner JF, Jones LW. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* 1979;17:16-23.
- Stonè KR, Mickey DD, Wunderli H, Mickey GH, Paulson DF. Isolation of a human prostate carcinoma cell line (DU 145). *Int J Cancer* 1978;21:274-81.
- Soule HD, Maloney TM, Wolman SR, et al. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res* 1990;50:6075-86.
- Jones HW Jr, McKusick VA, Harper PS, Wu KD, George Otto Gey. (1899-1970). The HeLa cell and a reappraisal of its origin. *Obstet Gynecol* 1971;38: 945-9.
- Drewinko B, Romsdahl MM, Yang LY, Ahearn MJ, Trujillo JM. Establishment of a human carcinoma-bryonic antigen-producing colon adenocarcinoma cell line. *Cancer Res* 1976;36:467-75.
- Gleave M, Hsieh JT, Gao CA, von Eschenbach AC, Chung LW. Acceleration of human prostate cancer growth *in vivo* by factors produced by prostate and bone fibroblasts. *Cancer Res* 1991;51:3753-61.
- Steinwaerder DS, Carlson CA, Lieber A. DNA replication of first-generation adenovirus vectors in tumor cells. *Hum Gene Ther* 2000;11:1933-48.
- Stanley PE. Commercially available luminometers and imaging devices for low-light level measurements and kits and reagents utilizing bioluminescence or chemiluminescence: survey update 5. *J Biolumin Chemilumin* 1997;12:61-78.
- Galanis E, Vile R, Russell SJ. Delivery systems intended for *in vivo* gene therapy of cancer: targeting and replication competent viral vectors. *Crit Rev Oncol Hematol* 2001;38:177-92.
- Siders WM, Halloran PJ, Fenton RG. Transcriptional targeting of recombinant adenoviruses to human and murine melanoma cells. *Cancer Res* 1996;56:5638-46.
- White E. Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. *Oncogene* 2001;20:7836-46.
- Jones N. Transcriptional modulation by the adenovirus *E1a* gene. *Curr Top Microbiol Immunol* 1995; 199:59-80.
- Hallenbeck PL, Chang YN, Hay C, et al. A novel tumor-specific replication-restricted adenoviral vector for gene therapy of hepatocellular carcinoma. *Hum Gene Ther* 1999;10:1721-33.
- Greenberg NM, DeMayo FJ, Sheppard PC, et al. The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. *Mol Endocrinol* 1994;8:230-9.
- DeWeese TL, van der Poel H, Li S, et al. A phase I trial of CV706, a replication-competent, PSA selective oncolytic adenovirus, for the treatment of locally recurrent prostate cancer following radiation therapy. *Cancer Res* 2001;61:7464-72.
- Jung C, Ou YC, Yeung F, Frierson HF Jr, Kao C. Osteocalcin is incompletely spliced in non-osseous tissues. *Gene* 2001;271:143-50.
- Rubinchik S, Lowe S, Jia Z, Norris J, Dong J. Creation of a new transgene cloning site near the right ITR of Ad5 results in reduced enhancer interference with tissue-specific and regulatable promoters. *Gene Ther* 2001;8:247-53.
- Doronin K, Kuppaswamy M, Toth K, et al. Tissue-specific, tumor-selective, replication-competent adenovirus vector for cancer gene therapy. *J Virol* 2001;75: 3314-24.
- Halbert DN, Cutt JR, Shenk T. Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff. *J Virol* 1985;56:250-7.
- Huang MM, Hearing P. Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection. *J Virol* 1989;63:2605-15.
- Richardson WD, Westphal H. A cascade of adenovirus early functions is required for expression of adeno-associated virus. *Cell* 1981;27:133-41.
- Weinberg DH, Ketner G. Adenoviral early region 4 is required for efficient viral DNA replication and for late gene expression. *J Virol* 1986;57:833-8.
- Dix I, Leppard KN. Regulated splicing of adenovirus type 5 E4 transcripts and regulated cytoplasmic accumulation of E4 mRNA. *J Virol* 1993;67: 3226-31.
- Freyer GA, Katoh Y, Roberts RJ. Characterization of the major mRNAs from adenovirus 2 early region 4 by

- cDNA cloning and sequencing. Nucleic Acids Res 1984;12:3503-19.
42. Tigges MA, Raskas HJ. Splice junctions in adenovirus 2 early region 4 mRNAs: multiple splice sites produce 18 to 24 RNAs. J Virol 1984; 50:106-17.
43. Virtanen A, Gilardi P, Naslund A, LeMoullec JM, Pettersson U, Perricaudet M. mRNAs from human adenovirus 2 early region 4. J Virol 1984; 51:822-31.
44. Ji L, Bouvet M, Price RE, Roth JA, Fang B. Reduced toxicity, attenuated immunogenicity and efficient mediation of human p53 gene expression *in vivo* by an adenovirus vector with deleted E1-E3 and inactivated E4 by GAL4-TATA promoter replacement. Gene Ther 1999;6:393-402.
45. Bristol JA, Zhu M, Ji H, et al. *In vitro* and *in vivo* activities of an oncolytic adenoviral vector designed to express GM-CSF. Mol Ther 2003; 7:755-64.
46. Kirn D, Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: Biological principles, risk management and future directions. Nat Med 2001;7: 781-7.
47. Harrison D, Sauthoff H, Heitner S, Jagirdar J, Rom WN, Hay JG. Wild-type adenovirus decreases tumor xenograft growth, but despite viral persistence complete tumor responses are rarely achieved—deletion of the viral E1b-19-kD gene increases the viral oncolytic effect. Hum Gene Ther 2001;12:1323-32.